Masaryk University

Faculty of Science

PROTEOMICS IN MOLECULAR ONCOLOGY AND IN BIOTECHNOLOGY

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Habilitation thesis

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TABLE OF CONTENTS

1 PREFACE

This habilitation thesis is based on scientific publications to which I have contributed as the corresponding author, first author and co-author. The articles were published between 2006 and 2016. All these publications are based on quantitative proteomics as a key tool to answer complex biological questions. With an original background in bacterial proteomics, I have been extending my research to the field of cancer research since 2004 and have established research direction of our group focused mainly on breast cancer metastasis, its biomarkers and mechanisms potentially targetable by the treatment. Proteomics remained a major research approach and, along with a development of proteomics technology, it has involved all key principles from gel based proteomics to the latest high-throughput and targeted proteomic tools. Proteomic tools have been complemented by additional cellular and molecular biology approaches.

After a general introduction to proteomics techniques, the thesis is divided into two blocks focused on proteomics in molecular oncology and biotechnology, respectively. Each block is further subdivided into 2-4 parts containing overview of the literature, problem definition and overview of our contribution to the field. The molecular oncology block covers the identification of potential metastatic biomarkers in breast cancer, their clinical validation using targeted proteomics, proteomics based cancer classification and chapter on biomarkers for cancer detection and non-invasive therapy monitoring. The biotechnology block is focused on applications of proteomics in metabolism control and stress response mechanisms in denitrifying bacteria and for studying the metabolism of sulfur oxidizing bacteria with biotechnological application.

The habilitation thesis is based on 18 publications in journals with impact factor (Supplement J1-J18). In addition, we have reviewed some more details in additional student's publications in journals indexed in Scopus and I add some of them to the thesis to provide a complete picture of our work (Supplements S1-S5), together with two published book chapters (Supplements C1-C2).

On August 25, 2016, I am author or co-author of 24 published papers in journals with impact factor, 13 papers in journals indexed in Scopus, 8 additional papers in other peer-reviewed journals, 2 chapters in books and 71 conference contributions.

2 INTRODUCTION

Proteins are the key structural, functional and catalytic units that form molecular complexes, cell organelles, cells and organs. In medicine, number of proteins play a crucial role as disease biomarkers that are associated with prognosis (prognostic biomarkers) and treatment decisions (predictive biomarkers) [1]. Central dogma of molecular biology defines how the genes encode the genetic information, how the genes are expressed and how the transcripts serve as information templates for proteins [2, 3]. There are number of regulation mechanisms and factors that define the level of proteins in the cells: (i) regulation of transcription, (ii) regulation of translation, (iii) transcript stability, (iv) posttranslational modifications of proteins, and (v) protein degradation (Fig. 1). Due to these reasons, correlation of transcripts and proteins is not perfect. It is estimated that it does not generally exceed ~40 % although higher percentage of agreement was shown for steady-state [4]. Most of high-throughput biology studies have been performed on transcript level (Affymetrix arrays, RNA-Sequencing), however, imperfect protein-transcript correlation makes the analysis of proteins essential. The reason is obvious: proteotype (a complex proteome profile) reflects the phenotype much better than genotype or transcriptome profile (Fig. 1).

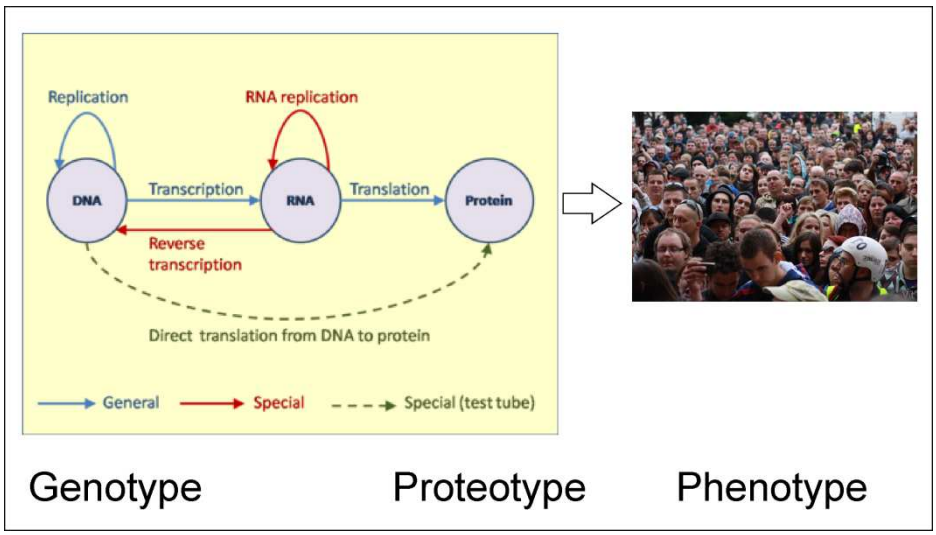

Fig. 1. Central dogma of molecular biology (yellow box) defines the mechanisms of gene expression *via* transcripts to proteins. Proteins (and not genes or transcripts) as the true structural molecules and regulatory effectors that form the phenotypes.

3 PROTEOMICS, A TOOL FOR COMPLEX PROTEIN ANALYSIS

Proteomics represents a methods toolbox to study proteins in a large scale. These methods have largely developed in recent years: Classical biochemical methods are able to bring new information on individual proteins and regulation of their levels. Development of mass spectrometry (MS) based technologies has provided new opportunities to study the complex protein composition of cells, tissues and biological fluids in their complexity with high accuracy and both sample and analyte throughput in last two decades. We have provided a complete overview of proteomics approaches in molecular medicine in a recently published book chapter [5] (Supplement C1).

3.1 TWO-DIMENSIONAL GEL ELECTROPHORESIS-MASS SPECTROMETRY BASED PROTEOMICS

The classical, namely in the past broadly used proteomics approach is based on two-dimensional gel electrophoresis (2-DE) that allows separation of hundreds to thousands proteins according to isoelectric point (pI) and molecular weight [6, 7] (Fig. 2). The individual proteins, their isoforms and posttranslational variants separated as spots can be quantified using image analysis. Selected spots can be digested with a protease (typically trypsin), resulting peptides can be extracted from the gel, ionized and fragmented in mass spectrometer (MS). Both ionized peptides (precursor ions) and peptide fragments (product ions) are measured in MS either in matrix assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) mode, or using liquid chromatography on-line connected to mass spectrometry with electrospray ionization (LC-ESI-MS). While the 2-DE method itself was published in 1975 [8], it was more broadly adopted after 1994 when the term “proteome” was defined by Marc Wilkins as a complex protein composition of cell, tissue or biological fluid [9] and when soft mass spectrometry ionization methods, MALDI-TOF MS and ESI-MS became available for routine peptide and protein identification. The current advantage of 2-DE based approach is that whole proteins (including their isoforms and posttranslational variants) and not their surrogate peptides are analyzed and quantified. However, since separation power of gels is not perfect, latest highly sensitive mass spectrometers are able to identify more than one

protein in each protein spot in the gel in some cases. This may lead to loss of link between protein identification and quantification and motivates the researchers to use more advanced methods.

We previously reviewed 2-DE-MS approach in [6] and used in 7 rather earlier articles involved in this thesis (Supplements J5, J13, J14, J16, J17, J18 and C2).

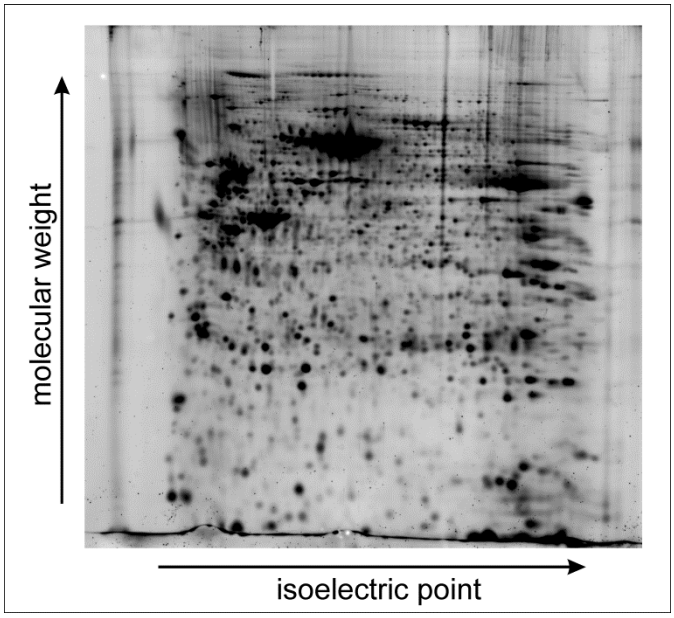

Fig. 2. Two-dimensional proteome map of breast cancer tissue. Each protein, its isoform or posttranslational variant is potentially visible as single spot, depending on its concentration and separation power of the gel. Protein quantification is based on image analysis. Proteins can be identified *via* mass spectrometry.

3.2 SURFACE ENHANCED LASER DESORPTION IONIZATION TIME-OF-FLIGHT MASS SPECTROMETRY

Surface enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF MS) is a relatively simple method that involves protein pre-separation on chips of various chemistries and detection and quantification of binding proteins in MALDI-TOF MS spectra [10]. This very high-throughput method enables measurement of up to few hundreds of samples per day in which whole undigested proteins are detected and quantified. The major disadvantage originates from low mass resolution of the MALDI-TOF mass analyzer. As a result, no more than few hundreds of polypeptides can be screened in single analysis, based on sample complexity. Another key disadvantage of SELDI-TOF MS approach is that identity of protein peaks is basically unknown and multidimensional fractionations on individual basis are required to obtain the protein identity of the peaks [11].

We used SELDI-TOF MS approach in 5 earlier articles involved in this thesis (Supplements J8, J9, J10, J11, J12).

3.3 SHOTGUN PROTEOMICS

Along with development in mass spectrometry technology, 2-DE-MS and SELDI-TOF have been replaced for many applications with more powerful liquid chromatography - mass spectrometry approach based on peptide sequencing and quantitation, called shotgun proteomics. Shortly, peptides obtained by digestion of proteins in complex samples using protease are typically separated with one- or multidimensional chromatography prior to tandem mass spectrometry (MS/MS) analysis. MS has been developing in term of sensitivity, resolution and speed to increase number of proteins that can be detected and quantified in single analysis. Orbitrap and quadrupole-time-of-flight (qTOF) are currently the most powerful mass analyzers used for untargeted proteomics analyses [12-14]. Shotgun proteomics currently allows identification and quantification of thousands proteins from complex sample. Quantification is ensured by labelling *via* isobaric tags for relative and absolute quantification (iTRAQ) [15], tandem mass tags (TMT) [16], dimethyl labelling [17], or stable isotope labelling in cell culture (SILAC) [18]. Alternatively, label-free quantification in MS1 mode can be used [19]. Detection of post-translational modifications (such as phosphorylation, glycosylation) is also possible from tandem mass spectra. Disadvantage of the approach originates from the fact that intact molecular weight of the whole proteins is not measured. It results in limited protein sequence coverage by identified peptides, loss of labile PTMs, and ambiguity of the origin for redundant peptide sequences [20]. This limits the information on isoforms and status of posttranslational modifications in the intact proteins.

We used shotgun proteomics in four articles involved in this thesis (Supplements J2, J3, J5 and J15).

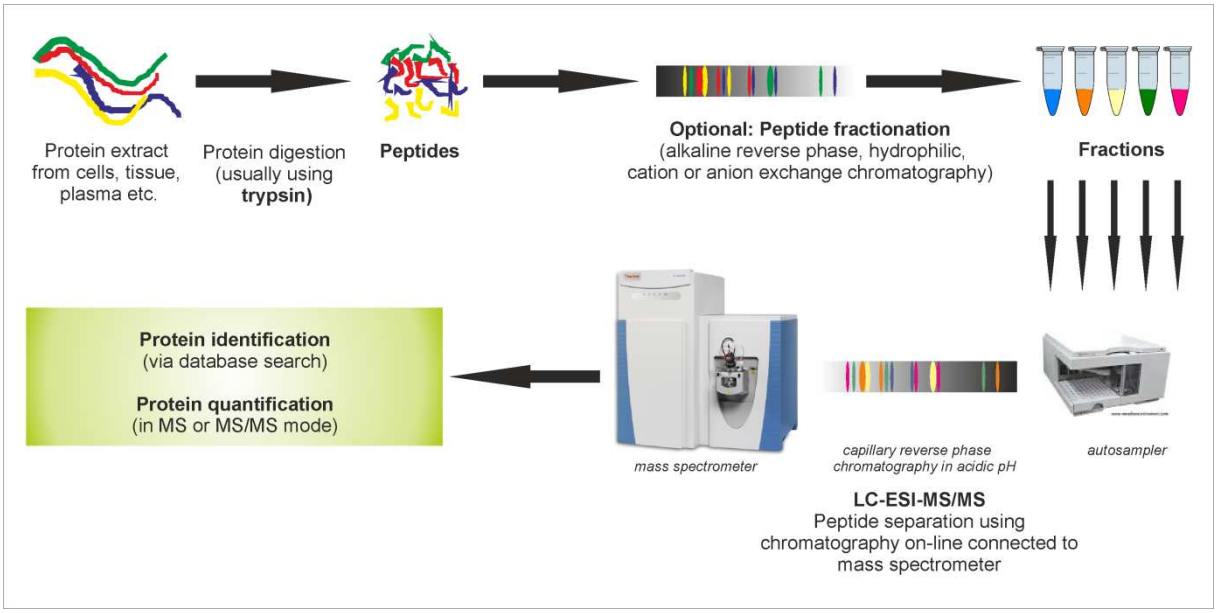

Fig. 3. LC-MS proteomics analysis of proteolytic peptides. Proteins are digested with protease (e.g., trypsin) to peptides, optionally fractionated and injected to capillary LC column on-line connected to mass spectrometer. Here, m/z and signal intensities of peptides and their fragmentation products are measured in data dependent ("shotgun" proteomics) mode: E.*g.*, 20 most intense precursor ions eluted at the particular time from the column are fragmented and their MS/MS spectra are obtained. Peptides and proteins are identified by database search of resulting MS/MS data. Alternatively, data acquisition can be performed in targeted or data independent modes, as explained in Fig. 4.

3.4 TARGETED PROTEOMICS

All above described approaches have been typically used for screening as many proteins as possible between biological states to find biologically important differentially regulated proteins and potential disease biomarkers. Sample throughput of discovery proteomics based on 2-DE or multidimensional LC-MS/MS is however relatively low. To validate such proteins in control experiments and in independent sets of samples, selected reaction monitoring (SRM) is a suitable approach with higher sample throughput. SRM has been well established in the analysis of small molecules and later on it was adopted for analysis of peptides and proteins -see Fig. 4 for basic principle. Development of SRM assays typically requires MS/MS spectra of peptides, from which the best combinations of precursor peptide ions and their fragments (called transitions) are selected and used in SRM assays for protein quantification. Isotopically labelled peptides are often used as internal standards for relative and/or absolute quantitation [21]. SRM assays are typically measured using triple-quadrupole mass spectrometer, however, alternative approaches on high-resolution mass

spectrometers such as pseudo-SRM (p-SRM or MRM^{HR}) [22] or parallel reaction monitoring (PRM) [23, 24] are now available.

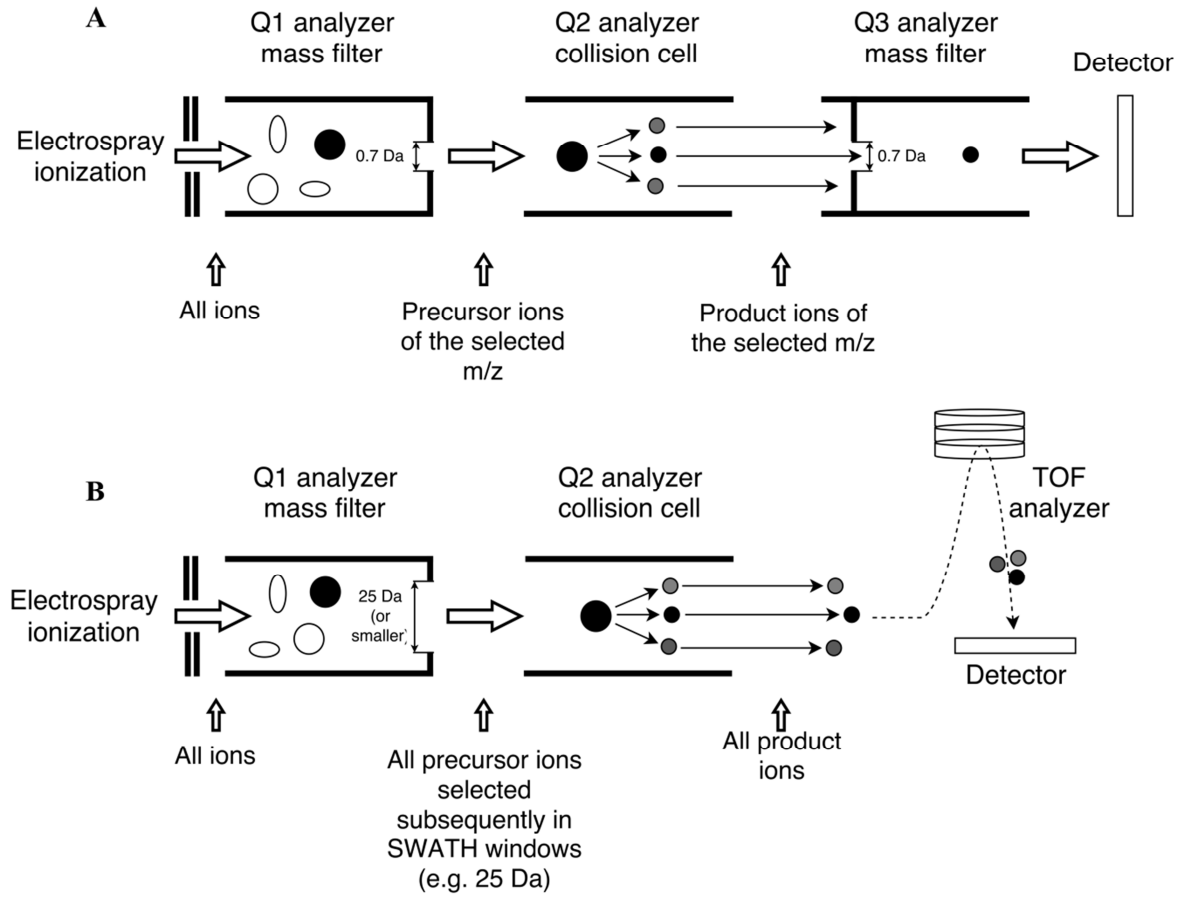

Fig. 4. Principles of targeted proteomics analysis. Peptides obtained by protein trypsin digestion are separated by liquid chromatography on-line connected to mass spectrometer (similarly to Fig. 3), in which data is obtained in different acquisition modes: **A**: In triple quadrupole (QqQ) mass spectrometer operating in SRM mode, precursor ions selected in Q1 (mass filter typically set to unit resolution, 0.7 Da) are fragmented in collision cell (Q2) to product ions. The third quadrupole (Q3) filters only selected product ions used for quantification. **B**: In mass spectrometer working in DIA mode (e.g., SWATH), the first analyzer selects ions using broader m/z windows (*e.g.*, 25 Da wide SWATH windows). Selected precursor ions are fragmented in collision cell and all product ions are analyzed in high resolution mass analyzer [25].

Data independent acquisition (DIA) methods represent multiplexed variant of SRM. One of the most commonly used approach, "sequential window acquisition of all theoretical fragment ion spectra mass spectrometry" (SWATH-MS) was published in 2012 by Gillet *et al*. [26]. In SWATH, not individual precursor ions but the whole effective mass range is being selected for fragmentation in sequential SWATH windows (Fig. 4), resulting in quantitative information on all detectable peptides in the sample, called digital fingerprints of the proteome. DIA typically enables consistent

quantitation of several thousands of proteins per ~2 h run with analytical characteristics comparable to SRM and data structure similar to SRM. One of the key advantages of SWATH is that the targets (peptides and proteins) are defined for targeted extraction of quantitative information in post-acquisition step while for SRM they have to be defined in acquisition method prior to the analysis. Although DIA is slightly less sensitive than SRM, it is evident that digital proteome maps obtained by DIA proteomics could serve for verification of protein levels *via* data extraction from publicly available datasets in the future, not requiring new data acquisition [25, 27].

We recently reviewed the principles of SRM in [28] (Supplement J6) and other targeted proteomics methods in [29] (Supplement S4). Two additional articles that involve targeted proteomics [25, 30] (Supplements J5, J7) are part of this thesis.

3.5 TOP-DOWN VS. BOTTOM-UP PROTEOMICS

As mentioned above, "old-fashioned" 2-DE-MS [31] an SELDI-TOF MS have been mostly replaced by modern shotgun and targeted proteomics with better proteome coverage and quantification accuracy. However, shotgun and targeted proteomics methods analyze tryptic peptides (bottom-up approach), while 2-DE and SELDI-TOF MS quantify whole undigested proteins (top-down approach). Top-down approach dramatically improves information on splice variants and posttranslational modifications of intact proteins. Moreover, it bypasses the need for proteolysis that typically increases the sample complexity and measurement irreproducibility due to proteolytic inhibition phenomena by molecules present in the clinical samples. This is a key advantage over most of current proteomics approaches based on shotgun proteomics [11]. Unfortunately, obtaining high-throughput top-down proteomics data is still technically difficult and limited to specialized research labs with an advanced experience and ultrahigh mass accuracy instruments, nevertheless, high-throughput top-down proteomics represents highly promising approach for the future [32].

One article in this thesis [11] (Supplement J9) is focused specifically on top-down proteomics field.

4 AIMS OF THE THESIS

Proteomics in molecular oncology

1. Identification, verification and validation of potential protein biomarkers associated with lymph node metastasis in breast cancer with major focus on low-grade tumors of luminal A subtype.

2. Verification of the above breast cancer biomarkers using targeted proteomics.

3. Classification of breast cancer tissues based on proteomic profiles and comparison of the results with transcript profiling.

4. Identification of biomarkers for cancer detection and non-invasive therapy monitoring.

Proteomics in biotechnological applications

1. Large-scale studying the mechanism of denitrification regulation *via* FNR-family transcription regulators and redox stress response in denitrifying bacteria.

2. Analysis of proteome dynamics of sulfur oxidizing bacteria in response to various growth conditions.

5 PROTEOMICS IN MOLECULAR ONCOLOGY

5.1 PROTEOMICS BASED IDENTIFICATION AND CHARACTERIZATION OF PROTEIN BIOMARKERS ASSOCIATED WITH BREAST CANCER METASTASIS

Breast cancer is the most common form of cancer in women worldwide. Currently, cancer metastasis and not the presence of primary tumor in the patient are the main reasons for patient mortality [33]. From the molecular point of view, cancer is multifactorial and multistage process that emerges as a consequence of multiple genetic aberrations. The same applies to cancer metastasis: The metastatic characteristics of cancer cells may be predisposed or acquired during disease development and are governed by a number of genetic and biochemical mechanisms [33, 34].

From the mechanistic point of view, metastasis formation involves dissemination of cancer cells from a primary tumor site to distant organs and tissues [35, 36] and their adaptation to novel tissue microenvironments [37] (Fig. 5). This process is called metastatic cascade and metastases are its terminal products [37, 38].

The first step of metastatic cascade represents the escape of cancer cells from primary tumor [38]. By overexpression of the proteases by both the cancer cells and the cells of the tumor environment, cancer cells break the basement membrane (Fig. 5b) and enter the stroma [37]. Stromal cells enhance aggressive behavior (invasiveness and migration potential) of cancer cells by production of cytokines and growth factors. These factors, together with hypoxia in tumor microenvironment, can trigger epithelial-to-mesenchymal transition (EMT) [39, 40]. During this process cancer cells remodel their cytoskeleton, lose cell-cell adhesion, lose epithelial phenotype and markers (*e.g.*, E-cadherin) and gain mesenchymal phenotype and produce its markers (*e.g.*, N-cadherin, vimentin). This leads to increased invasion and motility of these cells. Various signaling pathways like Wnt, Notch, NF-κB, TGF-β and other are also involved in EMT [41].

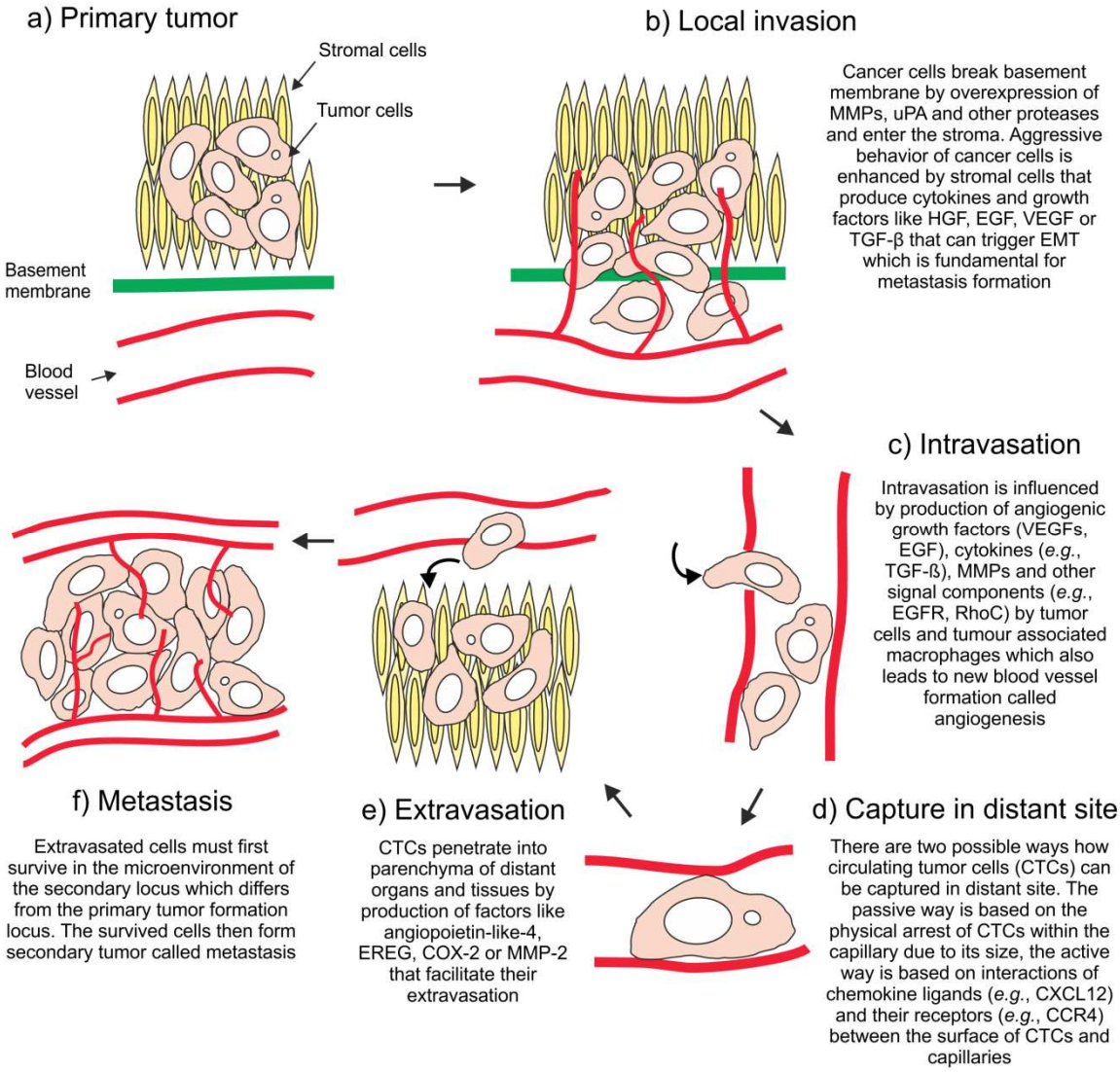

Fig. 5. Metastatic cascade is the process that consists of local invasion of cells from primary tumors through basement membrane and stromal cell layers (a-b); their intravasation into blood or lymph vessels (c); arrest at distant tissues and organs (d); extravasation into parenchyma of these tissues and organs (e); settlement and neoplastic growths of metastatic tumors (f) [37, 42, 43].

Next, locally invasive cancer cells penetrate into lumina of lymphatic or blood vessels. This process called intravasation is strongly influenced by tumor associated blood vessels [37], tumor cells and tumor associated macrophages which produce factors contributing to remodeling of extracellular matrix (ECM) [38] (Fig. 5c). New blood vessels formed during tumor progression are very penetrable that makes the intravasation easier [37]. After intravasation, circulating tumor cell (CTCs) must survive in spite of variety of stresses in the bloodstream before they achieve distant organ sites [37]. First of all, CTCs avoid specific type of apoptosis known as anoikis [40] which is triggered by

disruption of the integrin-dependent adhesion to ECM components. CTCs overcome degradation by innate immune system [37] by formation of metastatic thrombi between cancer cells and blood platelets [44]. Survived CTCs are transported by blood into distant organ sites. Whether the capture of CTCs in distant sites is passive [37] or active process [40] (Fig. 1d) must be first elucidated. Not all the cancer cells that successfully escape from their native tissue survive in circulation and grab in capillaries are able to extravasate or, in other words, to overcome physical barrier of endothelial cells and pericite layers by production of factors that induce vascular hyperpermeability and facilitate extravasation of tumor cells (Fig. 5f) [37, 38]. The great challenge for extravasated and thus disseminated carcinoma cells is to survive in secondary loci. The microenvironment at the metastatic site is usually different from that in primary tumor [37], but it has been proposed that primary tumors produce factors that contribute to appropriate environment in metastatic locus [40]. Even if the disseminated cancer cells survive, they are still not guaranteed to proliferate. Majority of extravasated cancer cells thus remain in long-termed dormancy as microcolonies and only small amounts are able to form macroscopic metastases [37].

In general, metastatic cascade is diverse but also highly inefficient process, especially in the later steps. The fact that metastases are the main cause of death in patients with solid malignancies [35] predicts the pro-metastatic pathways as sources of important therapeutics and diagnostics targets that become subjects of further studies [37, 43].

In clinical practice, both traditional and molecular prognostic markers have been used for risk-group discrimination and determination of metastatic potential. Traditional prognostic markers in breast cancer involve age at diagnosis, tumor size and grade, lymph node status and the presence of distant metastasis. Tumor size is a potent prognostic factor predicting higher probability of metastatic behavior for larger tumors. More differentiated tumors (*e.g.*, grade 1) have low dissemination potential in general, while less differentiated, more proliferative high grade tumors (*e.g.*, grade 3) form metastases much more frequently. Low grade breast tumor cells spread predominantly *via* lymph vessels and lymph nodes are therefore the first site of tumor cell

dissemination prior to their eventual spread into distant organs such as lung or bone [45]. Molecular prognostic markers involve hormonal receptors (estrogen receptor (ER), progesterone receptor (PR)), Her-2/neu receptor and expression panels like Oncotype DX and MammaPrint. Also, the American Society for Clinical Oncology (ASCO) has recommended urokinase plasminogen activator (PLAU) and urokinase plasminogen activator inhibitor (SERPINE1) as indicative factors for metastatic potential in breast cancer [46, 47], however their use in clinical practice has not been generally accepted [46]. There is thus a clinical need to find novel biomarkers that would identify patients with high risk of metastasis development who should receive more personalized therapy [48].

Currently available markers are not sufficient for precise risk-group or individual assessment specifically in low grade luminal-A tumors, whose general prognosis is very favorable, resulting in treatment by less aggressive adjuvant therapy and no chemotherapy. However, a low percentage of these tumors develop early lymph node metastases. The molecular mechanism of this phenomenon is not known and current clinical practice lacks the means for predicting its occurrence. New knowledge is thus essential for identifying biomarkers that can identify high risk individuals within the predominantly low risk population of patients with low grade breast cancers. These high risk patients should then receive more intensive follow-up and could be considered for more aggressive therapy, which cannot be achieved currently in view of the detrimental effects of therapy on the majority of patients who will not show benefit. In addition, understanding the mechanisms of metastasis of low grade breast cancer may lead to the identification of new therapeutic targets.

Our contribution to the field

The field of metastatic cascade, mechanisms, metastatic biomarkers, and contribution of proteomics to their investigation was reviewed in an invited paper published in special issue "Reviews 2014" of the journal PROTEOMICS [43] (Supplement J1). We have shown that both 2-DE and shotgun proteomics has enabled the identification of number of metastasis-associated proteins and potential biomarkers in cancer tissues, microdissected cells, model systems, and secretomes.

Proteomics has also contributed to functional description of such targets, either as a part of functional studies, or by interactome analysis. It is thus evident that current proteomics has a great potential to provide functionally meaningful data in the field [43]. We also published another complementary review more focused on the method approaches, including overview of methods to interactome analysis and identification of cell surface proteins related to metastasis [49] (Supplement S1).

Our first experimental study in the field was a pilot study performed and published in an international collaboration with dr. Spiros Garbis at Academy of Athens, Greece in 2009 [50] (Supplement J2). We firstly used a quantitative iTRAQ-2LC-MS/MS based proteomic method for the analysis of breast cancer tissue and its lymph node metastasis. We identified several differentially regulated proteins up-regulated in metastatic primary tumor and/or lymph node metastasis relative to the non-metastatic tumor type, namely: heme binding protein 1, group of interferon induced proteins, carbonic anhydrase 2 and parathymosin. On the other hand, some proteins involved in biosynthesis of lipids were down-regulated. The most important findings were verified also on transcript level. Our analysis confirmed applicability of iTRAQ-2DLC-MS/MS analysis in breast cancer biomarker discovery [50]. This method paper was cited 53x (Web of Science, August 2016). After publication, it was selected as the best publication in the field of proteomics in 2008-9 in the Czech Republic by Czech Society of Biochemistry and Molecular Biology in 2010 and received Arnold Beckman Prize.

A subsequent large study on clinically-pathologically representative set of 96 breast cancer patient tissues was performed and published in the journal Molecular and Cellular Proteomics in 2015 [48]. To identify markers for this group of patients, we employed a combined proteomics and transcriptomics to 24 lymph node positive and 24 lymph node negative grade 1 luminal A primary breast tumors. Another group of 48 high-grade tumors (luminal B, triple negative, Her-2 subtypes) was also analyzed to investigate marker specificity for grade 1 luminal A tumors (see Fig. 6 for the

workflow). The total of 4405 proteins were identified using an iTRAQ-2DLC-MS/MS proteomics (FDR<5%). The expression of top 65 differentially expressed together with 30 previously identified and control markers were analyzed also on transcript level. We identified a panel of proteins associated with lymph node metastasis in luminal A grade breast cancer and determined their specificity: Increased levels of carboxypeptidase B1 (CPB1), PDZ and LIM domain protein 2 (PDLIM2) and ring finger protein 25 (RNF25) were associated specifically with lymph node positive grade 1 tumors, whereas stathmin (STMN1) and thymosin beta 10 (TMSB10) associated with aggressive tumor phenotype also in high grade tumors at both protein and transcript level. Central transcription regulator of NF-κB pathway, p65/RELA, TRAF3 interacting protein 1 (TRAF3IP2) and 14-3-3η (YWHAH) were associated with lymph node status without delimited specificity. Protein level validation for CPB1 using immunohistochemical analysis on tissue microarrays showed similar differences. Additional transcript level validation in independent dataset of 343 patients was successful for CPB1 (p=0.00155), PDLIM2 (p=0.02027) and RELA (p=0.00015). Moreover, statistically significant connections with patient survival were identified in another public dataset (N=1678). Our findings indicated unique pro-metastatic mechanisms in grade 1 tumors that can include up-regulation of CPB1 and activation of NF-κB pathway [48]. At the time of publishing, this was the largest proteomics study on clinicopathologically representative set of breast cancer. Later on, additional studies were published by leading teams in highly impacted journals such as Nature [51] (by Clinical Proteomics Technology for Analysis of Cancer (CPTAC) consortium), Nature Communications [52] (by Matthias Mann group) or in Journal of Proteome Research [53] (by Emma Nimeus and Ruedi Aebersold teams) [54], however, none of them have focused on specifically metastatic biomarkers in luminal A tumors.

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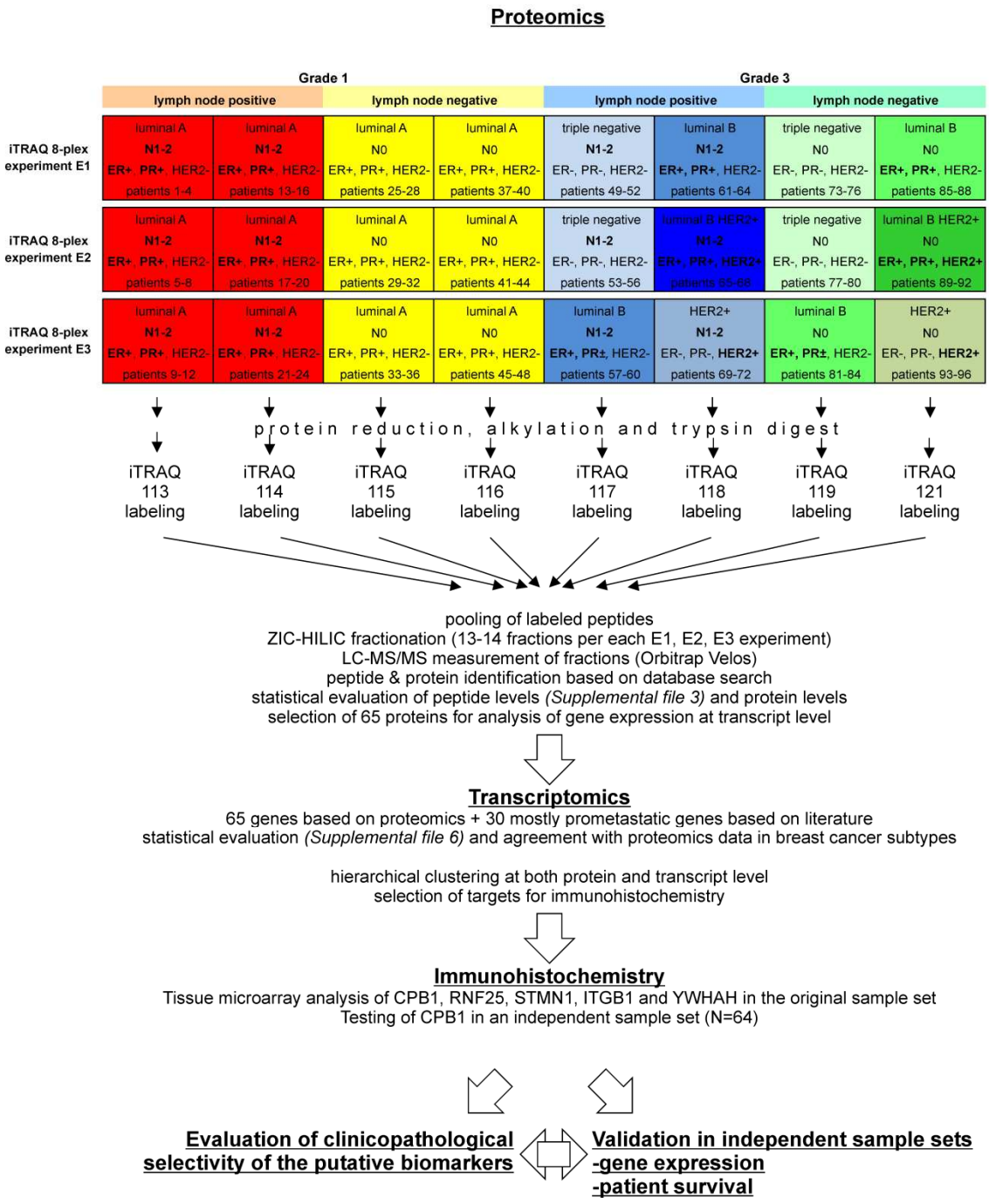

Fig. 6. Experimental design of a proteomic-transcriptomics study focused on identification of biomarkers of lymph node metastasis in low-grade breast cancer [48]. The design of proteomics experiment allowed analysis of 24 samples (each pooled from 4 patients' lysates, 24x4=96 patients involved); these 24 samples are represented by 24 rectangles in three rows corresponding to three iTRAQ 8-plex experiments (E1, E2, E3). Each pooled sample in each of three 8-plex experiments was labeled by one of eight iTRAQ labels (113, 114, 115, 116, 117, 118, 119, 121). For more details and more detailed legend see Supplement J3.

Results of our study led to hypothesis whether NF-κB activation in metastatic tumors could be targetable *via* NF-κB modulating anti-metastatic therapeutic strategy. Interestingly, it was

presented during San Antonio Breast Cancer Symposium (Dec 8-12, 2015) that receptor activator of nuclear factor-kappa B (RANK) inhibitor denosumab improves disease-free survival for postmenopausal patients with hormone receptor positive breast cancer according to the results of the phase III ABCSG-18 clinical trial. Moreover, the data suggest that this treatment should be offered to all patients with hormone receptor positive breast cancer who are receiving adjuvant aromatase inhibitor therapy, irrespective of their bone health status [55]. Although this data does not allow making conclusions on possible effect on metastasis development, it indicates that NF-κB blocking is an effective approach in early breast cancer treatment, supporting findings of our recent combined proteomics-transcriptomics study [48].

One of the proteins identified in our above mentioned study [48] was PDLIM2. It is a member of the actinin-associated LIM family of proteins that play essential roles in cytoskeleton organization, cell differentiation and have been associated with oncogenesis. Josef Maryáš *et al*. have reviewed the current knowledge on PDLIM2 role in cancer development [56] (Supplement S2). Shortly, it is known that PDLIM2 regulates stability and activity of several transcription factors, *e. g*. NF-κB or STAT, and its deregulation is associated with several malignancies. Interestingly, PDLIM2 expression has been connected with both tumor suppression and tumorigenesis: PDLIM2 levels are epigenetically suppressed in different cancers due to Mystique promoter hypermetylation that blocks its transcription, moreover, PDLIM2 re-expression is able to inhibit tumorigenicity and induces tumor cell death both *in vitro* and *in vivo*, which suggest potential tumor suppressor role of PDLIM2. On the other hand, PDLIM2 is highly expressed in cancer cell lines derived from metastatic cancer and its expression is associated with tumor progression and metastasis formation, indicating pro-oncogenic role of PDLIM2. In our review, we hypothesized a dual model of this protein function in early and late stage of tumorigenesis that could explain the contradictory observations [56]. Our latest experimental results show its pro-metastatic role by influencing migration, invasion and EMT of breast cancer cells (Maryas J. *et al*., in preparation), fully supporting our previous expression results in clinical cohorts.

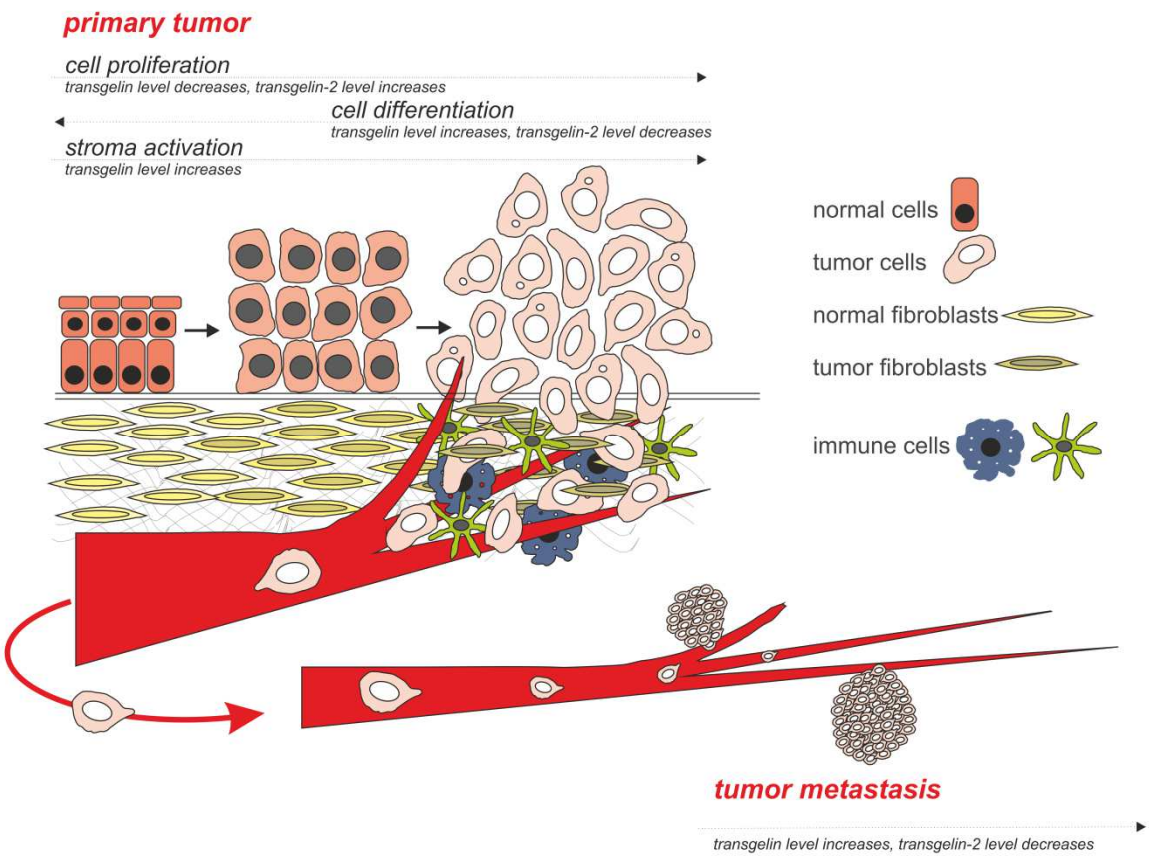

Fig. 7. Overview of transgelin and transgelin-2 expression in relation to the important processes in cancer. It seems that transgelin and transgelin-2 levels change conversely during tumor development. Whilst transgelin expression is detected mainly in the cells of tumor stroma, where its level increases together with stroma activation and decreases together with dedifferentiation of the tumor cells, transgelin-2 is restricted to the tumor cells where its level increases together with dedifferentiation of the tumors and thus with a tumor progression. On the other hand results of some studies indicate that transgelin level positively correlates with the metastatic potential of the tumor cells [58].

Our research has also focused on the group of cytoskeletal proteins, transgelins. Transgelin was firstly identified as protein associated with lymph node metastasis of breast cancer in very early phase of our research by Jarmila Sobotkova using 2-DE [57]. Transgelin is an abundant protein of smooth muscle cells, where its role has been primarily studied. As a protein affecting dynamics of the actin cytoskeleton *via* stabilization of actin filaments, transgelin is both directly and indirectly involved in many cancer-related processes such as migration, proliferation, differentiation or apoptosis. Monika Dvořáková proceeded in this research direction, preparing a comprehensive review on the role of transgelins in cancer [58] (Supplement J4). We summarized contradictory

observations in the literature and reviewed the role of transgelin in tumors (see also Fig. 7), concluding that expression and biological role of transgelin seems to differ among various types of tumor cells and stroma and possibly change during tumor progression. This first comprehensive review on transgelin function in cancer has received around 20 manuscript requests and 10 citations from other authors since 2014.

Our experimental work on transgelin association with lymph node metastatis in breast cancer was summarized in a paper published in Journal of Proteomics in 2016 [30] (Supplement J5). Initial transgelin discovery in the cohort of 12 patients using 2-DE-MS was followed by a combined proteomics and transcriptomics validation in the set of 96 breast cancer tissues. The validation data has confirmed transgelin connection with the lymph node metastasis and, moreover, immunohistochemical staining specified that transgelin is expressed mostly in tumor stroma. These results entered the research into the topical field of carcinoma associated fibroblasts [59, 60] as discussed in the paper. Our data on transgelin homologue, transgelin-2, show that it was mainly expressed by epithelial cancer cells and its levels were increased in metastatic and poorly differentiated tumors [30] (Supplement J5). Subsequent functional analyses of BT549 and PMC42 breast cancer cell lines with silenced transgelin expression *vs*. control cell lines indicate connection of transgelin with cell migration and apoptosis in breast cancer (Dvořáková M. *et al*., in preparation).

Important step in studying a pro-metastatic role of identified proteins is analysis of their contribution to cell migration and invasiveness. There are several *in vitro* methods available: They include simple assays such as scratch–wound assay and methods based on chemotaxis (*e.g.,* Dunn‘s chamber). More complex methods that enable studying both cell migration and invasiveness include systems based on Boyden chamber such as Transwell migration/invasion test, xCELLigence system, and confocal microscopy based approaches. *In vivo* methods are represented mainly by mice model. Petra Kovaříková has used *in vitro* methods in her diploma thesis [61] to study the role of carboxypeptidase B1 (CPB1) in migration and invasiveness. Based on her experience, we reviewed

principles and summarized pros and cons of the methods in this toolbox [62] (Supplement S3).

5.2 VERIFICATION AND CLINICAL VALIDATION OF BREAST CANCER BIOMARKERS USING TARGETED PROTEOMICS

As shown above, proteomics holds a potential to find novel biomarkers for detection, diagnosis and prognosis of diseases including cancer. To overcome potential method issues such as peptide co-elution in LC-MS, it is recommended to verify proteomics findings using an independent method. More importantly, to overcome biological issues related to genetic, inter-sample and intra-sample (*e.g*. intra-tissue) heterogeneity, it is essential to validate the selected biomarker candidates in large independent sets of samples to confirm their clinical relevance. For these purposes, the availability of targeted methods for protein quantification with high sample throughput is very important. Immunoaffinity-based approaches such as immunohistochemistry (IHC) for tissues, or enzyme-linked immunosorbent assay (ELISA) for blood samples are not available for all biomarker candidates, especially for the new ones, and their development on-purpose is expensive and time consuming. Targeted proteomics represents a reasonable alternative, also because of faster method development. Many studies [27, 63-68] have proved good correlation of gold standard of targeted proteomics, SRM, with immunoaffinity-based methods suggesting that SRM is suitable for biomarker quantification and can complement traditional methods in cancer research. More recently, targeted data extraction from SWATH-MS datasets could serve for biomarker verification in publically available datasets not requiring new data acquisition in the future (reviewed in [27]). In a current literature, establishment of targeted proteomics assays, generation of assay databases such as SRM Atlas [69] and method application for routine protein determination in biological samples has been solved in most aspects [25]. This fact contributed to selection of targeted proteomics as “Method of the year 2012” by the journal Nature Methods [70].

Our contribution to the field

We reviewed the technical principles of selected reaction monitoring (SRM) and current

status of knowledge essential to assay development and targeted proteomics method building in [28](Supplement J6). We explained that the instrumentation for SRM-based targeted proteomics basically consists of a nano-flow liquid chromatograph coupled with (nano)electrospray ionization and a triple-quadrupole mass spectrometer. We described the functionality of the quadrupoles in protein applications, showed our first results on model samples, discussed the applications in medicine and outlined future directions in the field. The principles of targeted proteomics approaches on high-resolution mass spectrometers (pseudo-SRM, SWATH, HRM) were summarized elsewhere [29](Supplement S4), highlighting the differences to SRM by the use of time-of-flight detector (TOF) instead the third quadrupole (Q3) and its consequences. The aim of the article was also to show the potential and model applications of targeted proteomics to the clinicians involved in cancer research where it serves for studying the biologically important proteins and their role in cancer development, progression and metastasis.

The outputs of targeted proteomics research, both SRM-based and DIA-based, in the field of solid tumors were comprehensively overviewed by Vendula Pernikářová *et al*. [71] (Supplement J7). We have shown that targeted proteomics has been successfully used in a high number of studies answering clinical questions on solid malignancies, namely breast, colorectal, prostate, ovarian, endometrial, pancreatic, hepatocellular, lung, bladder and other cancers. We summarized that 62 potential biomarkers of breast cancer were collectively validated using targeted proteomics by 16 studies, 29 for colorectal cancer by 9 studies, 9 for prostate cancer by 11 studies, 27 for ovarian cancer by 7 studies, 2 for endometrial cancer by 3 studies, 14 for pancreatic cancer by 7 studies, 16 for hepatocellular cancer by 8 studies, 33 for lung cancer by 11 studies and 8 for bladder cancer by 2 studies. The potential biomarkers successfully quantified using targeted proteomics through large amount of studies are Erbb2/HER2, lactate dehydrogenase A and pyruvate kinase (PK) in breast cancer, S100A9 protein in colorectal cancer, prostate serum antigen (PSA) in prostate cancer, PK in endometrial cancer, a-1-acid glycoprotein, ceruloplasmin (CERU) and a-fetoprotein in hepatocellular cancer and serum amyloid A in lung cancer. Moreover, proteins clusterin, CERU, aldolase A,

osteopontin, fibronectin and inter-a trypsin inhibitor heavy chain H3 (ITIH3) were quantified throughout many types of malignancies [71]. By overviewing verified biomarker candidates successfully quantified by targeted proteomics in the field, our review has directed the readers who plan to design their own hypothesis-driven experiments to appropriate sources of methods and knowledge.

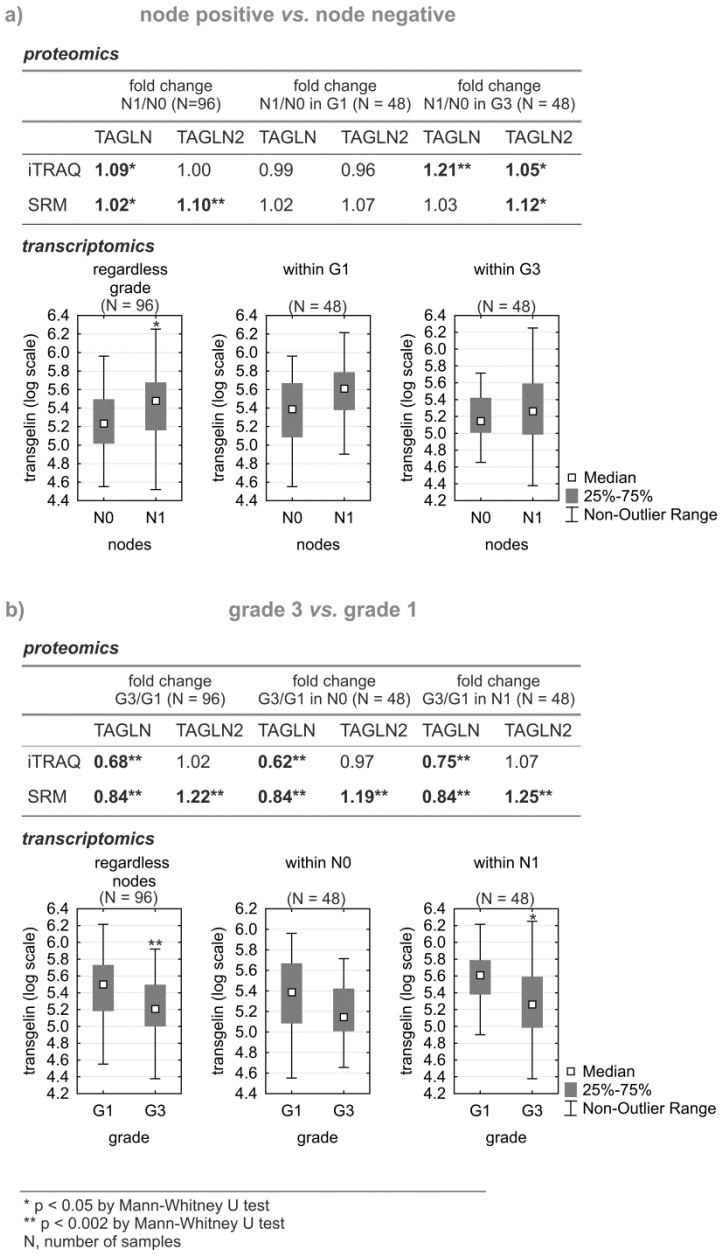

Fig. 8. Differential expression of transgelin (TAGLN)/transgelin-2 (TAGLN2) according to lymph node status (part a) and tumor grade (part b). Using iTRAQ-proteomics, targeted proteomics and transcriptomics, we determined significant increase of both proteins in lymph node positive (N1) vs. lymph node negative (N0) tumors and significant decrease of transgelin and increase of transgelin-2 level in grade 3 (G3) tumors in comparison with grade 1 (G1) tumors. Transgelin mRNA level is expressed as normalized absolute number of mRNA copies in log scale [30].

We firstly experimentally used targeted proteomics to verify differences in levels of transgelins in breast cancer tissues. We developed a SRM assay with mass differential tags for

relative and absolute quantification (mTRAQ) labelling (mTRAQ-SRM) for transgelin and transgelin-2 proteins and analyzed the clinically-pathologically representative set of 96 breast cancer tissues. Our SRM results confirmed transgelin connection with the lymph node metastasis observed by shotgun proteomics and transcriptomics and showed that both proteins have been reversely connected to tumor grade and tumor cell differentiation [30] (Supplement J5). Similar mTRAQ-SRM analyses have been performed for additional 11 proteins (AGR2, ANXA1, STMN1, COF1, PAIRBP1, TSP2, POSTN, ACTN4, RNF25, GELS, BGH3) in the same set of 96 breast cancer tissues, highlighting a panel of validated proteins connected to several aspects of breast cancer aggressiveness (estrogen receptor status, tumor grade and lymph node status) that are of further interest in terms of diagnostics and/or more precise breast cancer treatment ([72], under review).

5.3 BREAST CANCER CLASSIFICATION *VIA* PROTEOMICS

As mentioned above in part 5.1, breast cancer is a disease relatively well characterized on molecular level [52]. Despite progress in early diagnosis and therapy, many patients however develop fatal disease. Breast cancer is currently classified into five subtypes or immunophenotypes based on cell proliferation/tumor grade, estrogen receptor (ER), progesterone receptor (PR) and HER2 status. The immunophenotypes can be defined as luminal A (ER+, HER2-, low proliferation), luminal B (ER+, HER2-, high proliferation), luminal B HER2 positive (ER+, HER2+, high proliferation), HER2 subtype (ER-, HER2+, high proliferation) and triple negative (ER-, PR-, HER2-, high proliferation) [73]. This classification serves to guide decisions for first line therapy which, however, fails in a substantial proportion of cases due to cancer recurrence, therapy resistance and/or metastasis. Development of advanced, generalized disease despite first line therapy indicates that the current classification of tumors based on immunophenotyping may not fully reflect the genetic, inter- and intra-tumor heterogeneity. The search for better tumor classifiers nowadays concentrates on the application of omics approaches able to analyze thousands of genes, gene transcripts or proteins in

one experiment. Proteins are the direct effector molecules in the cell and their direct measurement is therefore preferable over transcript measurements (expression arrays, RNA sequencing) [74].

Our contribution to the field

In our early study published in 2008 [75] (Supplement J8), we analyzed a set of 105 tissue lysates of breast carcinomas using SELDI-TOF MS on IMAC 30 ProteinChip Arrays to study their classification based on proteomic profiles. Cluster analysis of protein spectra was performed to identify protein patterns potentially related to established clinicopathological variables and/or tumor markers. Unsupervised hierarchical clustering of 130 peaks detected in spectra from breast cancer tissue lysates provided six clusters of peaks and five groups of patients differing significantly in tumor type, nuclear grade, presence of hormonal receptors, mucin 1 and cytokeratin 5/6 or cytokeratin 14 (Fig. 9). These tumor groups resembled closely luminal types A and B, basal and HER2-like carcinomas. Our results showed similar clustering of tumors to those provided by cDNA expression profiles of breast carcinomas (Fig. 9). This fact testified the validity of proteomic approach in such a type of study. As proteomics provides different information from cDNA expression profiles, the results suggested the technique's potential to supplement and expand our knowledge of breast cancer, to identify novel biomarkers and to produce clinically useful classifications of breast carcinomas [75]. This paper was highlighted as highly accessed by the Breast Cancer Research journal upon publication.

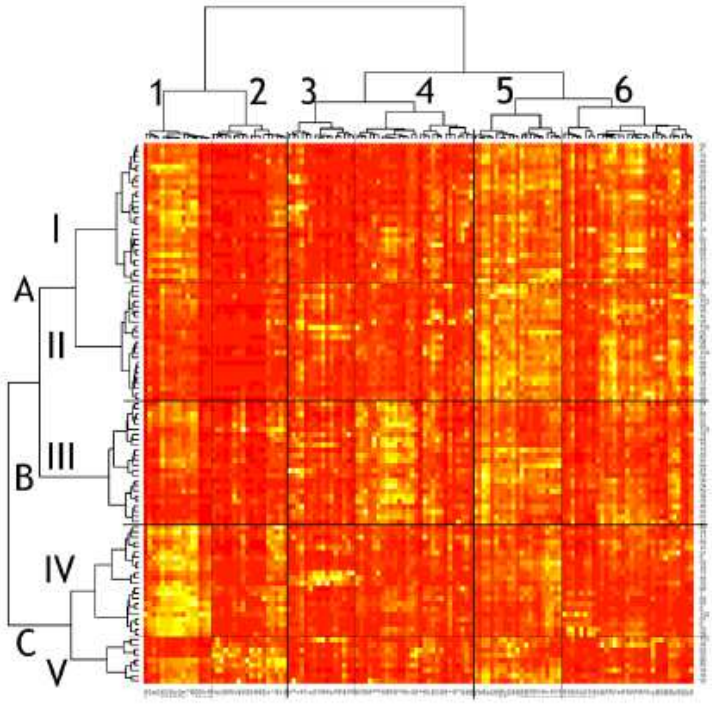

Fig. 9. Result of hierarchical clustering in the form of a heat map of peak values. Rows represent 105 individual patients and columns represent 130 peaks used for the analysis. The value of the peak is indicated by the color intensity. Unsupervised hierarchical clustering revealed two (not labeled), three (labeled A, B, C), five (labeled I to V) and six (labeled 1 to 6) groups of patients [75].

In 2013 we published a paper "Intact protein profiling in breast cancer biomarker discovery: protein identification issue and the solutions based on 3D protein separation, bottom-up and top-down mass spectrometry" [11] (Supplement J9) focused on a new protocol based on 3DLC-MS/MS with top-down proteomics approach. The new method enabled to obtain an identity of protein peak of m/z=3357 that correlated with estrogen receptor positivity in breast cancer tissues in the previous study [75] as an intact non-tryptic peptide originating from HNRNP A2/B1 protein. Importantly, we summarized our experience on multidimensional separation techniques for identification of intact proteins screened using SELDI-TOF MS and discussed aspects of future development of this general concept towards high-throughput, top-down based protein profiling at high resolution.

Another paper in the field of proteomics classification of breast cancer using SWATH-MS method is currently in preparation in collaboration with Prof. Ruedi Aebersold at Institute of Molecular Systems Biology at ETH Zürich, Switzerland [74].

5.4 IDENTIFICATION OF BIOMARKERS FOR NON-INVASIVE CANCER DETECTION AND THERAPY MONITORING

Number of proteomics studies has utilized tissue samples, cell cultures and *in vivo* model systems in the past to identify potential biomarker candidates of cancer. To enable non-invasive diagnostics based on secreted tissue markers, it is essential to analyze the profiles of these proteins also in bodily fluids such as plasma and serum. In principle, the marker proteins secreted by the cancer tissue might enter the blood where they could be potentially determined as serum markers of the disease [76]. This determination of high clinical need is, however, significantly complicated (i) by protein dilution in 5-6 L of human blood decreasing the marker concentration and increasing requirements on method sensitivity for serum/plasma quantification, (ii) by another tissues that may secrete the same protein into blood [77]. Another issue is the presence of contaminants of non-protein nature that may affect the mass profile of the sample. This is especially important for MS-only based techniques where the identity of the peaks is unknown, such as SELDI-TOF MS. Moreover, some of these contaminants may originate from blood collection devices [78].

Our contribution to the field

Clot-activating blood-sampling devices are routinely used in clinical laboratories for serum chemistry and immunoassay testing because they provide serum within a short period of time and may reduce turnaround times. However, it has not been sufficiently demonstrated that these tubes are free from interferences. In our study [79] (Supplement J10), we collected 2 fasting blood specimens from each of 20 healthy female volunteers. Specimens were collected in 2 types of tubes: Microvette Sarstedt, type neutral, denoted “white”, and Microvette Sarstedt Serum Gel Clotting activator, denoted “brown”.

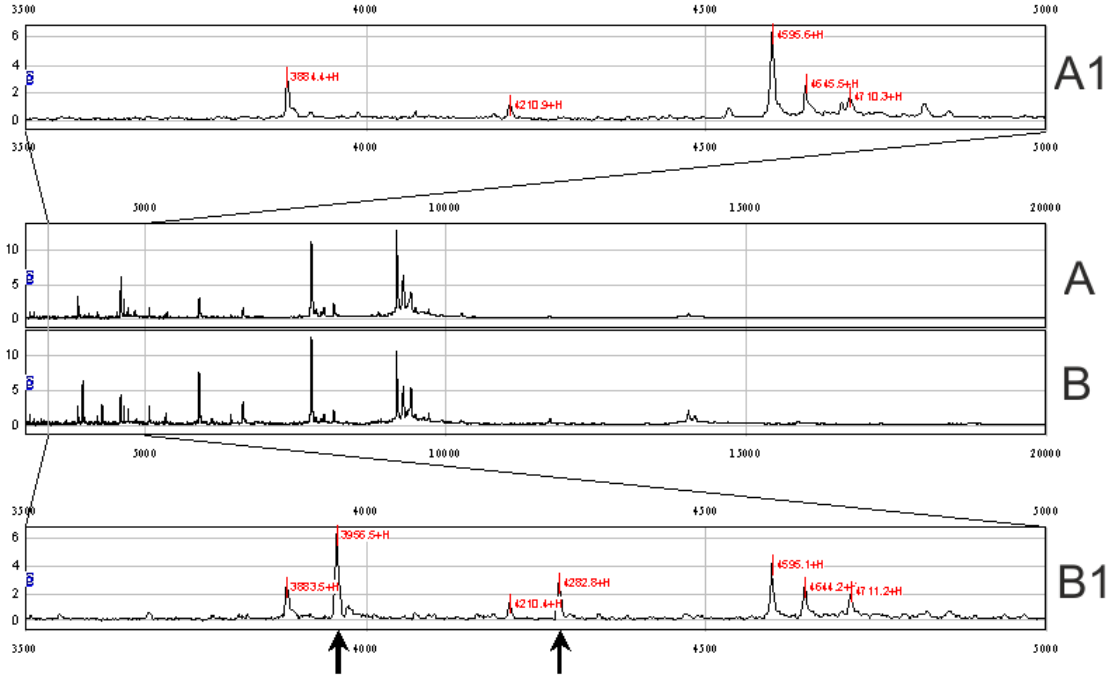

Fig. 10. Typical SELDI/TOF MS spectra of blood samples collected into plain tube (A and A1-cutout) and gel clotting activator containing tube (B and B1-cutout). The peaks with area increasing after blood collection into gel clotting activator containing tube are marked with arrows in spectra cutouts (B1 vs. A1)[79].

Within the mass range analyzed (3000–20 000 *m/z*) by SELDI-TOF MS, 34 peak clusters were found. In the brown sample group, we detected 2 peaks, *m/z* 3957.3 and 4283.6, with intensities whose mean normalized peak areas were >40-fold higher than those of the white sample group. These peaks were situated next to 2 peaks with *m/z* 3885.2 and 4211.2, the signal of which was decreased in some spectra in the brown group (Fig. 10). It seems that more rapid coagulation process in clot-activator tubes may be associated with more extensive proteolysis in the specimen, potentially leading to greater protein digestion that is subsequently detected by mass spectrometry. Non-biological changes, observed repeatedly in the low-molecular-weight serum proteome profiles, raised the question of whether serum is the specimen of choice for major protein- and/or peptide-type clinical analytes such as hormones and tumor markers. In summary, we have shown that clot activator-containing collection tubes may lead to pre-analytical artifacts in proteomic studies. In our experience, these tubes can be effectively substituted with Li-heparin plasma tubes for chemistry analytes or plain serum tubes used for immunoassay and specimen banking [79].

In the study by Holcakova *et al*. [80] (Supplement J11), we applied SELDI-TOF MS technology

to study tissues from renal cell carcinomas (RCC) in comparison to healthy controls. We found that different biomarkers are identified from tissues than those previously identified in serum, and that serum markers are often not produced by the tumors themselves at detectable levels, reflecting the non-specific nature of many circulating biomarkers. We detected and characterized αB-crystallin as an overexpressed protein in RCC tissues and showed differential expression by immunohistochemistry. We conclude that SELDI-TOF is more useful for the identification of biomarkers that are synthesized by tumor tissues than for the identification of serum biomarkers and identifies a separate set of markers. We suggest that SELDI-TOF should be used to screen human cancer tissues to identify potential tissue-specific proteins and simpler and more sensitive techniques can then be applied to determine their validity as biomarkers in biological fluids [80].

Proteomics can be also tested for identification of proteins that are connected with levels of administered drugs. We complemented an initial pharmacokinetic study of a new anticancer agent (OC-6-43)-bis(acetato)(1-adamantylamine)amminedichloroplatinum (IV) (LA-12) by proteomic screening of rat plasma. The objective of our proteomics study [81] (Supplement J12) was to identify new LA-12 target proteins that serve as markers of LA-12 treatment, response and therapy monitoring. Proteomic profiles were measured by SELDI-TOF MS in 72 samples of rat plasma randomized according to LA-12 dose and time from administration. Correlation of 92 peak clusters with platinum concentration was evaluated using Spearman analysis. We identified Retinol-binding protein 4 (RBP4) whose level correlated with LA-12 level in treated rats. Similar results were observed in randomly selected patients involved in Phase I clinical trials. RBP4 induction was in agreement with known RBP4 regulation by amantadine and cisplatin. Since retinol metabolism is disrupted in many cancers and inversely associates with malignancy, this data identified a potential novel mechanism for the action of LA-12 and other similar anti-cancer drugs [81].

6 PROTEOMICS IN BIOTECHNOLOGICAL APPLICATIONS

6.1 PROTEOMICS IN STUDYING METABOLISM REGULATION AND CELL STRESS RESPONSE IN DENITRIFYING BACTERIA

Paracoccus denitrificans is a non-fermentative, facultatively autotrophic soil bacterium often studied in the field of bioenergetics, particularly due to the resemblance of its aerobic respiratory chain to that of mitochondria. It has a potential of biotechnological application in nitrate removal in waterworks, in waste water treatment and in agriculture. This bacterium responds to a decreasing level of oxygen and the presence of nitrate in the environment by a switch from aerobic to a denitrification growth mode. The first step of denitrification is the reduction of nitrate to nitrite, which is then sequentially reduced *via* nitric oxide (NO) and nitrous oxide to dinitrogen gas [82]. *P. denitrificans* is one of the organisms that expresses all four key enzymes of this process, *i.e*. the nitrate, nitrite, nitric oxide and nitrous oxide reductases [83]. Expression of these enzymes is tightly controlled on the transcription level not only globally according to an energetic hierarchy but also on the level of the individual genes. It ensures a proper balance in the concentration and activity of the reductases since the intermediate compounds of denitrification, nitrite and nitric oxide, are cytotoxic [84]. The major players in this regulatory network are three members of the FNR (fumarate and nitrate reductase regulatory protein) family of transcription regulators, which upon activation by their corresponding signals bind to specific sites (FNR boxes) in target promoters upstream of the σ factor binding site to stabilize the RNA-polymerase transcription initiation complex (reviewed in [85]). One of the paralogues is FnrP which has a [4Fe-4S] cluster for oxygen sensing [86], the second is NNR, which has a heme for NO sensing [87] and the third one is NarR which is poorly characterized and likely to be a nitrite and nitrate sensor [88, 89]. All three have dedicated tasks in gene regulation and cannot take over each other's role [90]. In response to oxygen deprivation, FnrP controls the expression of the *nar* gene cluster encoding nitrate reductase, the *cco*-gene cluster encoding a cbb_3-type oxidase for respiration at low oxygen concentrations and the *ccp* gene encoding cytochrome *c* peroxidase. NNR specifically controls the expression of the gene clusters encoding the nitrite (*nirS*),

and nitric oxide (*norCB*) reductases and, to a certain extent, nitrous oxide (*nosZ*) reductase. NarR is required for the transcription of the *nar* gene cluster in an unknown interplay with the FnrP protein [88, 91]. These properties have been deduced from a number of studies on each of these transcriptional activators, but knowledge on the interplay between these regulators along with their position in the complete regulatory network are scarce [94].

P. denitrificans is also a useful model for studying redox stress in bacterial cells. Living in an oxygenated environment brings about the possibility of a non-enzymatic one-electron reduction of molecular oxygen to the superoxide anion (O_2^-), a primary source of several other potentially dangerous oxygen metabolites known as reactive oxygen species (ROS). A useful tool for studying the mechanisms that cells employ to protect themselves from ROS is to provoke a cellular response by application of an exogenous ROS source. Aerobically growing *P. denitrificans* elaborate a respiratory chain closely resembling that of mitochondria with respect to the presence of the respiratory complexes I–IV and a ubiquinone homologue with ten isoprene units in the side chain (UQ-10). Besides the mitochondrial-type cytochrome aa_3 terminal oxidase, additional oxidases branch from the respiratory chain at the level of ubiquinone (cytochrome ba_3 oxidase) and cytochrome(s) *c* (cytochrome cbb_3 oxidase). In the absence of oxygen, energy is gained from denitrification catalyzed by denitrifying enzymes under control of the FNR proteins as described above. We recently determined the structure of a cytoplasmic flavoprotein termed FerB and studied its function [92]: In *in vitro* experiments it reduced ubiquinones (UQ-0, UQ-10) and superoxide and *in vivo* it provided partial protection against methyl viologen-induced lipid peroxidation [93]. We thus proposed that FerB may represent a prokaryotic counterpart of the well-known mammalian antioxidant enzyme NAD(P)H:quinone oxidoreductase 1 (NQO1) that plays a crucial role in defense against oxidative stress and hypothesized a similar role for FerB.

Our contribution to the field

In our first related paper involved in this thesis [94] (Supplement J13), we analyzed the

protein composition of four *P. denitrificans* strains (wild type, FnrP-, NNR- and NarR-mutant strains) grown aerobically, semiaerobically and semiaerobically in the presence of nitrate to discover the global role of FNR-family transcription regulators using proteomics, with data validation at the transcript and genome levels. Expression profiles were acquired using two-dimensional gel electrophoresis for 737 protein spots, in which 640 proteins were identified using mass spectrometry. The annotated 2-D proteome map provided the most comprehensive coverage of *P. denitrificans* proteome available to-date and can be accessed on-line at http://www.mpiib-berlin.mpg.de/2D-PAGE/ (see [95], Supplement C2, for details).

Our results revealed several types of regulation under the conditions tested (Fig. 11): (1) FnrP-controlled regulation of the nitrous oxide reductase, UspA and OmpW confirmed at protein, transcript and DNA level (position of FNR boxes). (2) Proteins regulated via additional regulators, including proteins involved in NNR and NarR regulons: nitrate reductase β-subunit, TonB-dependent receptors, nitrite reductase, a TenA-type transcription regulator, and an unknown protein with an alpha/beta hydrolase fold. (3) Proteins whose expression was affected mainly by the growth condition. This group contains a SSU ribosomal protein S305 /σ54 modulation protein, and two short-chain reductase–dehydrogenase proteins [94]. After the publication, FnrP-controlled regulation of nitrous oxide reductase reported in this study attracted further attention in the group Asa Fostergard and Linda Bergaust at Norvegian University of Life Sciences who followed our proteomics screening by more targeted experiments and reported that nitrous oxide reductase regulation also responds to pH [96], oxygen and nitric oxide *via* both FnrP and NNR transcription regulators [97].

Our previous proteomics study [98] showed that *P. denitrificans* cells undergo changes in protein composition upon exposure to azide, a known activator of the FNR-type transcription factor NarR. One of the most prominent protein species inducible by azide is a Fe/Mn-family superoxide dismutase (SOD) [99](Supplement J14). Azide induced SOD at protein, mRNA transcript and enzyme activity levels in the aerobically growing cells. Since SOD expression remains unaffected in the *fnrP*,

nnr and *narR* mutant strains, we postulated a mechanism independent of the known FNR-type regulators but involving a redox signal arising from the respiratory chain [99].

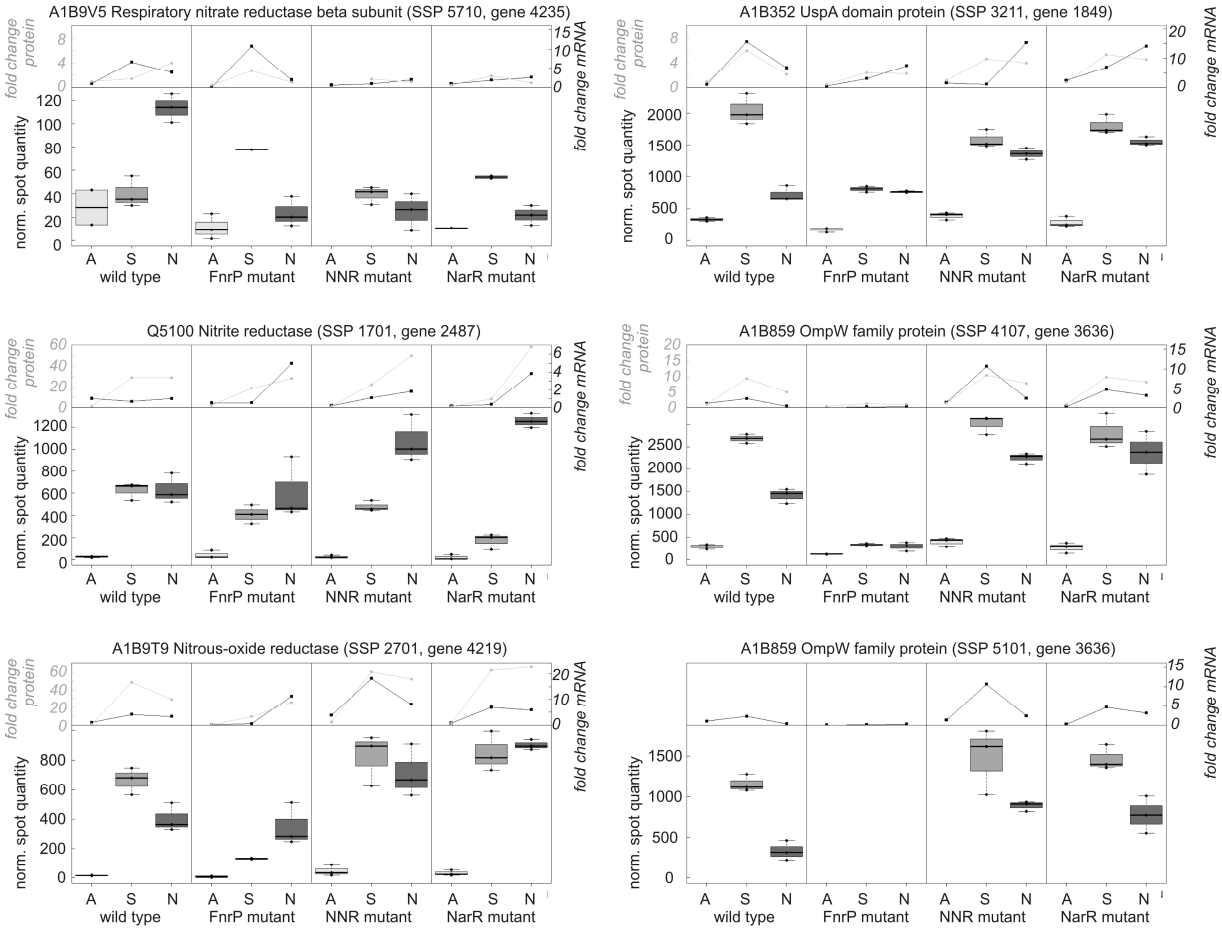

Fig. 11. Expression profiles of proteins whose gene promoters possess FNR box sequences. Downregulation in FnrP mutant in the cases of nitrous oxide reductase, UspA and OmpW proteins indicate direct regulation via FnrP protein; expression profiles of these proteins are highly correlative [94].

Based on our previous work [92], we hypothesized that FerB in *P. denitrificans* may play a putative role in defense against oxidative stress. To further explore this hypothesis, we compared protein variations upon methyl viologen treatment in wild-type and FerB mutant strains by a quantitative proteomics based on iTRAQ-3DLC-MS/MS approach. This comprehensive analysis resulted in identification of 24948 peptides representing 2627 proteins (FDR<0.01) in *P. dentirificans* wild type and *ferB* mutant strains (54% coverage of the theoretical proteome) grown in the presence or absence of methyl viologen as an oxidative stressor (see [100], Supplement S5 for dataset details). The proteins showing the most prominent increase in abundance were assigned to carbon fixation and sulfur assimilatory pathways. By employing these proteins as indirect markers, oxidative stress

was found to be 15 % less severe in the wild-type than in the FerB-deficient mutant cells. Oxidative stress altered the levels of proteins whose expression is dependent on the transcriptional factor FnrP. The observed down-regulation of the *fnrP* regulon members, most notably that of nitrous oxide reductase, was tentatively explained by an oxidative degradation of the [4Fe-4S] center of FnrP leading to a protein form which no longer activates transcription. While the level of FerB remained relatively constant, two proteins homologous to FerB accumulated during oxidative stress. When we expressed their genes in *Escherichia coli*, neither of the protein products contained a bound flavin, whereas they both had a high activity of flavin reductase, one preferentially utilizing NADH and the other NADPH [71] (Supplement J15).

6.2 PROTEOMICS IN STUDYING SULFUR OXIDIZING BACTERIA

Acidophilic microorganisms such as *Acidithiobacillus sp.*are chemolithotrophs that can oxidize ferrous iron and inorganic sulfur compounds. These bacteria are involved in formation of acid mine drainage and have strong potential in biotechnology, namely in biohydrometallurgy [101, 102]. Both iron- and sulfur-oxidizing cells play a different role in oxidation of metal sulfides. Specific proteins are expressed during iron or sulfur metabolism, and proteomic analysis can provide the quantitative and qualitative markers typical of iron or sulfur oxidation under both aerobic and anaerobic conditions. Since the genome of *A. ferrooxidans* was fully sequenced (http://www.tigr.org), there was a challenge to perform complex proteomic analyses of this environmentally and industrially important microbe [103].

Our contribution to the field

A first comparative analysis of the proteome of *A. ferrooxidans* cells with immobilized pH gradients and mass spectrometry protein identification was performed by us and published in the journal PROTEOMICS in 2006 [103](Supplement J16). A newly developed protocol involved also improved protein reduction, MALDI-MS and LC-MS/MS protein identification based on full genome sequence information. This approach resulted in more than 1300 protein spots displayed in broad

and basic pH ranges, the best *A. ferrooxidans* proteome resolution at the time of publication (Fig. 12). A comparative image analysis of cells grown on elemental sulfur and ferrous iron revealed that the proteome was significantly influenced by the growth type, and allowed for the detection of many physiologically important proteins. Among them were sulfate adenylyltransferase and sulfide dehydrogenase, which are involved in sulfate assimilation and sulfide metabolism, respectively. Many other proteins were related to important processes like cell attachment and electron transport. Co-migration of phosphate and sulfate transport proteins was also observed [103].

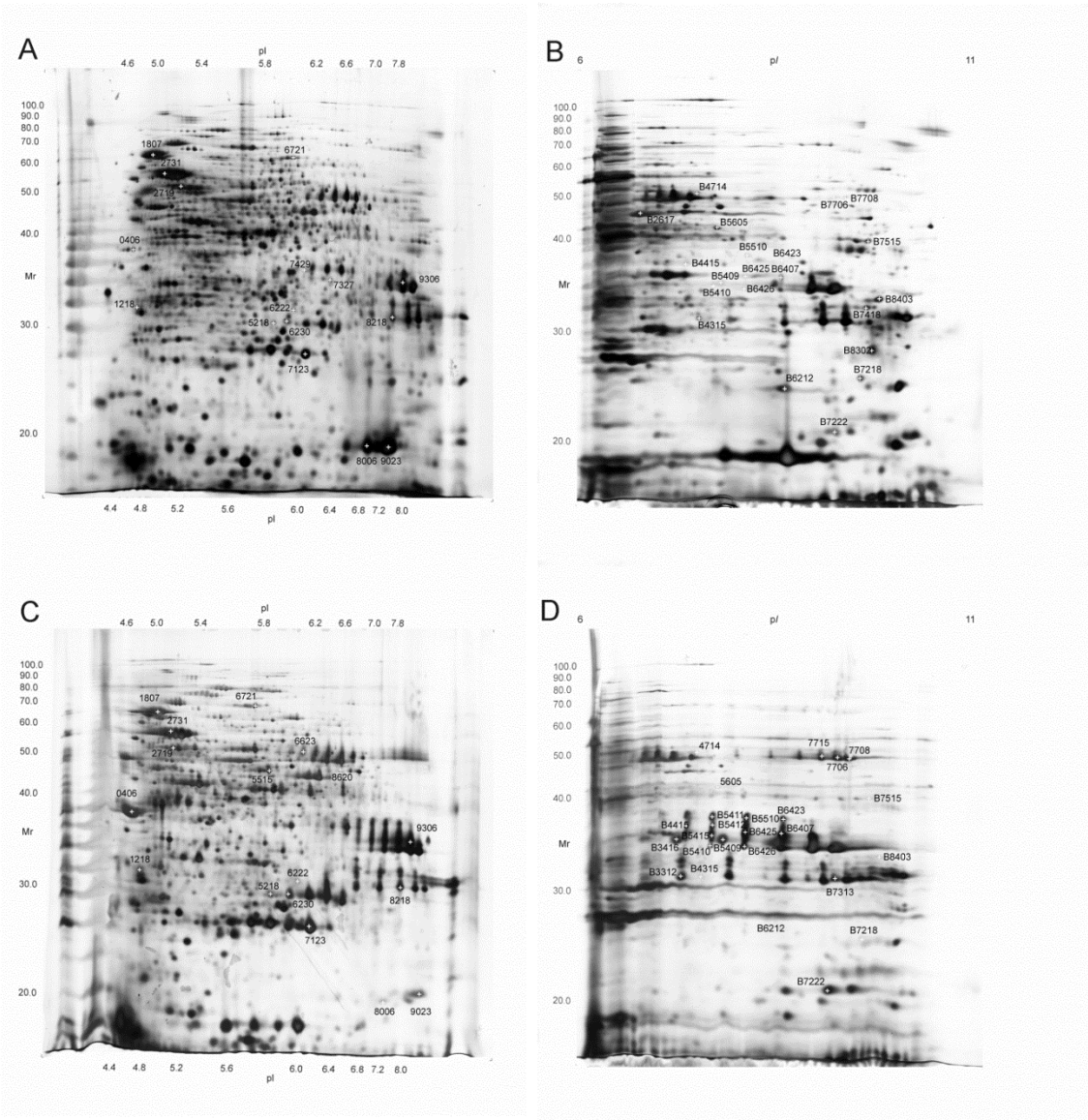

Fig. 12. Representative 2-DE proteome maps of *Acidithiobacillus ferrooxidans* CCM 4253 grown on ferrous iron (A, B) and elemental sulfur (C, D). Total cell lysate was analyzed using broad range (3–10 NL; A, C) and basic (6–11; B, D) pH gradients. At least five-fold differentially regulated protein spots between both growth modes were selected for MS identification; these spots are marked with spot numbers [103].

The first proteomic screening of total cell lysates of anaerobically incubated *A. ferrooxidans* resulted in the detection of 1599 protein spots in the master two-dimensional electrophoresis gel [104] (Supplement J17). A set of 59 more abundant and 49 less abundant protein spots that changed their protein abundances in an anaerobiosis-dependent manner was identified and compared to iron- and sulfur-grown cells, respectively. Proteomic analysis detected a significant increase in abundance under anoxic conditions of electron transporters, such as rusticyanin (rus) and cytochrome c_{552}, involved in the ferrous iron oxidation pathway. Therefore we suggested the incorporation of rus-operon encoded proteins in the anaerobic respiration pathway. Two sulfur metabolism proteins were identified, pyridine nucleotide-disulfide oxidoreductase and sulfide-quinone reductase. The important transcription regulator, ferric uptake regulation protein, was anaerobically more abundant. The anaerobic expression of several proteins involved in cell envelope formation indicated a gradual adaptation to elemental sulfur oxidation.

In contrast to iron-oxidizing *A. ferrooxidans, A. ferrooxidans* from a stationary phase elemental sulfur-oxidizing culture exhibited a lag phase in pyrite oxidation, which is similar to its behavior during ferrous iron oxidation [105] (Supplement J18). The ability of elemental sulfur-oxidizing *A. ferrooxidans* to immediately oxidize ferrous iron or pyrite without a lag phase was only observed in bacteria obtained from growing cultures with elemental sulfur. However, these cultures that shifted to ferrous iron oxidation showed a low rate of ferrous iron oxidation while no growth was observed. We used 2-DE-MS approach for a quantitative proteomic analysis of the adaptation process when bacteria were switched from elemental sulfur to ferrous iron. A comparison of total cell lysates revealed 39 proteins whose increase or decrease in abundance was related to this phenotypic switching. However, only a few proteins were closely related to iron and sulfur metabolism. Reverse-transcription quantitative PCR was used to further characterize the bacterial adaptation process. The expression profiles of selected genes primarily involved in the ferrous iron oxidation indicated that phenotypic switching is a complex process that includes the activation of genes encoding a membrane protein, maturation proteins, electron transport proteins and their regulators.

Nowadays, we have implemented label-free LC-MS proteomics in the analysis of acidophiles. Pilot data confirmed identification of new unique proteins whose expression was specifically dependent on substrates and culture conditions. Ongoing analyses indicate promising results that may lead to unravelling of the novel metabolic pathways under extreme conditions.

7 CONCLUSIONS

Proteomics is a systems biology tool to analyze proteins in a large scale. It was established in mid-nineties and involves gel-based, shotgun and targeted mass spectrometry approaches. Shotgun proteomics was shown to be well applicable in cancer biomarker discovery field to identify proteins associated with lymph node metastasis in breast cancer. We have identified carboxypeptidase B1 and NF-κB associated proteins as the key proteins connected with lymph node metastasis in luminal A breast cancer and validated them on transcript level and in independent datasets [48]. Based on these results, we hypothesized NF-κB pathway as a target of anti-metastatic therapy. To-date, this was indirectly confirmed by preliminary data of ABCSG-18 trial which showed that NF-κB blocking *via* denosumab is an effective therapeutic approach in hormone receptor positive early breast cancer [55]. Transgelin [30] was identified, validated and further characterized as additional potential marker for lymph node status in breast cancer. Tumor classification of breast cancer mostly recapitulated current clinicopathologically relevant subtypes and it is evidently effective to provide more comprehensive clinically useful classifications of this heterogeneous disease [75]. Although retinol binding protein 4 [81] was identified as potential marker for LA-12 treatment monitoring, proteomics identification of clinically applicable serum or plasma biomarkers remains challenging [79][80][71]. Proteomics has been successfully used in studies on metabolic regulation, redox stress and response to extreme growth condition in bacteria with potential biotechnological applications.

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9 SUPPLEMENTS: LIST OF PUBLICATIONS INCLUDED IN THE THESIS

Articles published in journals with impact factor

[J1] MARYÁŠ, Josef, Jakub FAKTOR, Monika DVOŘÁKOVÁ, Iva STRUHÁROVÁ, Peter GRELL and Pavel BOUCHAL. Proteomics in investigation of cancer metastasis: Functional and clinical consequences and methodological challenges. Proteomics, Wiley VCH, 2014, vol. 14, No. 4-5, p. 426-440. ISSN 1615-9853. doi:10.1002/pmic.201300264. IF=4.132
http://onlinelibrary.wiley.com/doi/10.1002/pmic.201300264/abstract
Contribution to the paper: Overall idea, supervision of whole manuscript preparation process and writing

[J2] BOUCHAL, Pavel, Theodoros ROUMELIOTIS, Roman HRSTKA, Rudolf NENUTIL, Bořivoj VOJTĚŠEK and Spiros D. GARBIS. Biomarker discovery in low-grade breast cancer using isobaric stable isotope tags and two-dimensional liquid chromatography-tandem mass spectrometry (iTRAQ-2DLC-MS/MS) based quantitative proteomic analysis. Journal of Proteome Research, Washington, USA: ACS, 2009, vol. 8, No. 1, p. 362-373. ISSN 1535-3893. doi:10.1021/pr800622b. IF=5.675
http://pubs.acs.org/doi/abs/10.1021/pr800622b
Contribution to the paper: First idea of biological question and method combination, development of tissue lysis method, proteomics and transcriptomics data interpretation, manuscript writing

[J3] BOUCHAL, Pavel, Monika DVOŘÁKOVÁ, Theodoros ROUMELIOTIS, Zbyněk BORTLÍČEK, Ivana IHNATOVÁ, Iva PROCHÁZKOVÁ, JTC HO, Josef MARYÁŠ, Hana IMRICHOVÁ, Eva BUDINSKÁ, Rostislav VYZULA, Spiros D. GARBIS, Bořivoj VOJTĚŠEK and Rudolf NENUTIL. Combined Proteomics and Transcriptomics Identifies Carboxypeptidase B1 and Nuclear Factor kappa B (NF-kappa B) Associated Proteins as Putative Biomarkers of Metastasis in Low Grade Breast Cancer. MOLECULAR & CELLULAR PROTEOMICS, BETHESDA: AMER SOC BIOCHEMISTRY MOLECULAR BIOLOGY INC, 2015, vol. 14, No. 7, p. 1814-1830. ISSN 1535-9476. doi:10.1074/mcp.M114.041335. IF= 7.254
http://www.mcponline.org/content/14/7/1814.short?rss=1
Contribution to the paper: Supervision of sample preparation for proteomics, contribution to proteomics data analysis, proteomics data interpretation, selection of targets for transcriptomics validation and qRT-PCR array design, transcriptomics data interpretation, selection of targets for immunohistochemistry and interpretation of results, analysis of patient survival data, paper writing

[J4] DVOŘÁKOVÁ, Monika, Rudolf NENUTIL and Pavel BOUCHAL. Transgelins, cytoskeletal proteins implicated in different aspects of cancer development. Expert Review of Proteomics, 2014, Vol. 11, No. 2, p. 149-165. ISSN 1478-9450. doi:10.1586/14789450.2014.860358. IF=3.896
http://www.tandfonline.com/doi/full/10.1586/14789450.2014.860358
Contribution to the paper: First idea, supervision of paper writing

[J5] DVOŘÁKOVÁ, Monika, Jarmila JEŘÁBKOVÁ, Iva PROCHÁZKOVÁ, Juraj LENČO, Rudolf NENUTIL and Pavel BOUCHAL. Transgelin is upregulated in stromal cells of lymph node positive breast cancer. Journal of Proteomics, Amsterdam: ELSEVIER SCIENCE BV, 2016, Vol. 132, No. 11, p. 103-111. ISSN 1874-3919. doi: 10.1016/j.jprot.2015.11.025. IF=3.888
http://www.sciencedirect.com/science/article/pii/S1874391915301986
Contribution to the paper: Supervision of 2-DE-based analysis and interpretation, design of validation phase, supervision of paper writing

[J6] FAKTOR, Jakub, Iva STRUHÁROVÁ, Alena FUČÍKOVÁ, Martin HUBÁLEK, Bořivoj VOJTĚŠEK a Pavel BOUCHAL. Kvantifikace proteinových biomarkerů pomocí hmotnostní spektrometrie pracující v režimu monitorování vybraných reakcí. Chemické listy, Praha: Česká společnost chemická, 2011, Vol. 105, No. 11, p. 846-850. ISSN 0009-2770. IF=0.717

http://www.chemicke-listy.cz/docs/full/2011_11_846-850.pdf
Contribution to the paper: Overall idea, supervision of whole manuscript preparation process and writing

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Contribution to the paper: Overall idea, supervision of whole manuscript preparation process and writing, "Expert commentary" part writing

[J8] BROŽKOVÁ, Kristýna, Eva BUDINSKÁ, Pavel BOUCHAL, Lenka HERNYCHOVÁ, Dana KNOFLÍČKOVÁ, Dalibor VALÍK, Rostislav VYZULA, Bořivoj VOJTĚŠEK and Rudolf NENUTIL. Surface-enhanced laser desorption/ionization time-of-flight proteomic profiling of breast carcinomas identifies clinicopathologically relevant groups of patients similar to previously defined clusters from cDNA expression. Breast Cancer Research, London, Great Britain: Biomed Central Ltd., 2008, Vol. 10, No. 3, p. R48. ISSN 1465-5411. doi:10.1186/bcr2101. IF=2.880
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Contribution to the paper: Method contribution to SELDI-TOF MS study, identification of selected protein peaks

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Contribution to the paper: Experimental design, method development, data interpretation and paper writing

[J10] PILNÝ, Radomír, Pavel BOUCHAL, Šárka BOŘILOVÁ, Pavla ČEŠKOVÁ, Jan ŽALOUDÍK, Rostislav VYZULA, Bořivoj VOJTĚŠEK and Dalibor VALÍK. Surface-enhanced laser desorption-ionization/time-of-flight mass spectrometry reveals significant artifacts in serum obtained from clot activator-containing collection devices. Clinical Chemistry, Washington, USA: Amer. Assoc. Clinical Chemistry, 2006, Vol. 52, No. 11, p. 2015-2016. ISSN 0009-9147. IF=7.717
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Contribution to the paper: Method contribution to SELDI-TOF MS study, data analysis, interpretation and paper writing

[J11] HOLČÁKOVÁ, Jitka, Lenka HERNYCHOVÁ, Pavel BOUCHAL, Kristýna BROŽKOVÁ, Jan ŽALOUDÍK, Dalibor VALÍK, Rudolf NENUTIL and Bořivoj VOJTĚŠEK. Identification of alphaB-crystallin, a biomarker of renal cell carcinoma by SELDI-TOF MS. The International Journal of Biological Markers, Itálie: Wichtig editore, 2008, Vol. 23, No. 1, p. 48-53. ISSN 0393-6155. IF=0.966
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Contribution to the paper: Method contribution to SELDI-TOF MS study, data analysis and interpretation

[J12] BOUCHAL, Pavel, Jiří JARKOVSKÝ, Kristýna HRAZDILOVÁ, Monika DVOŘÁKOVÁ, Iva STRUHÁROVÁ, Lenka HERNYCHOVÁ, Jiří DAMBORSKÝ, Petr SOVA and Bořivoj VOJTĚŠEK. The new platinum-based anticancer agent LA-12 induces retinol binding protein 4 in vivo. Proteome Science, BioMed Central, 2011, Vol. 9, No. 68. ISSN 1477-5956. doi:10.1186/1477-5956-9-68. IF=2.488
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Contribution to the paper: SELDI-TOF MS data analysis and interpretation, supervision of protein identification, supervision of immunochemical validation, paper writing

[J13] BOUCHAL, Pavel, Iva STRUHÁROVÁ, Eva BUDINSKÁ, Ondrej ŠEDO, Tereza VYHLÍDALOVÁ, Zbyněk ZDRÁHAL, Rob VAN SPANNING and Igor KUČERA. Unraveling an FNR based regulatory circuit in Paracoccus denitrificans using a proteomics-based approach. Biochimica et Biophysica Acta - Proteins and Proteomics, Amsterdam, The Netherlands: Elsevier, 2010, Vol. 1804, No. 6, p. 1309-1316. ISSN 1570-9639. doi:10.1016/j.bbapap.2010.01.016. IF=2.480
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Contribution to the paper: Bacterial cultivation, protein extraction, supervision of 2-DE and image analysis, proteomics and transcriptomics data interpretation, paper writing

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Contribution to the paper: supervision of proteomics and transcriptomics analysis and enzyme activity determination, manuscript writing

[J15] PERNIKÁŘOVÁ, Vendula, Vojtěch SEDLÁČEK, David POTĚŠIL, Iva PROCHÁZKOVÁ, Zbyněk ZDRÁHAL, Pavel BOUCHAL and Igor KUČERA. Proteomic responses to a methyl viologen-induced oxidative stress in the wild type and FerB mutant strains of Paracoccus denitrificans. Journal of Proteomics, Amsterdam: ELSEVIER SCIENCE BV, 2015, Vol. 125, July, p. 68-75. ISSN 1874-3919. doi:10.1016/j.jprot.2015.05.002. IF=3.888
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Contribution to the paper: iTRAQ-3DLC method optimization, supervision of proteomics analysis, MS data analysis and interpretation, contribution to manuscript preparation

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Contribution to the paper: Sample preparation development, 2-DE proteomics optimization, most of the interpretation, paper writing

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Contribution to the paper: Supervision of 2-DE proteomics analysis, contribution to proteomics data interpretation

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Contribution to the paper: Initial idea on PDLIM2 studying, supervision of the writing

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Contribution to the paper: Overall idea, supervision of the manuscript preparation process and writing

[S5] PERNIKÁŘOVÁ, Vendula, Vojtěch SEDLÁČEK, David POTĚŠIL, Iva PROCHÁZKOVÁ, Zbyněk ZDRÁHAL, Pavel BOUCHAL and Igor KUČERA. Proteome-wide dataset generated by iTRAQ-3DLCMS/MS technique for studying the role of FerB protein in oxidative stress in Paracoccus denitrificans. Data in Brief, Amsterdam: Elsevier, 2015, Vol. 4, September, p. 390-394. ISSN 2352-3409. doi:10.1016/j.dib.2015.06.015.
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Contribution to the paper: Dataset preparation for database, paper writing